



Diethyl pyrocarbonate inactivates CD39/ecto-ATPDase by modifying His-59

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Received 20 October 1999; received in revised form 9 February 2000; accepted 17 February 2000

Abstract

Diethyl pyrocarbonate (DEPC) in conditions that favour carbethoxylation of histidyl residues strongly inactivated E-type ATPase activity of a rat lung membrane preparation, as well as ecto-ATPase activity of rat vessels and human Epstein–Barr virus-transformed B lymphocytes. Inactivation of the enzyme (up to 70%) achieved at concentrations of DEPC below 0.5 mM could be fully reversed by 200 mM hydroxylamine at pH 7.5, thus confirming histidine-selective modification. UTP effectively protected the enzyme activity from DEPC inactivation. This was taken to indicate that the conformation adopted by the enzyme molecule upon substrate binding was not compatible with DEPC reaching and/or modifying the relevant histidyl residue. Substrate activation curves were interpreted to show the enzyme molecule to be inactive, at all substrate concentrations tested, when the target histidyl residue had been modified by DEPC. Comparison of known sequences of CD39-like ecto-ATP(D)ases with the results on inactivation by DEPC revealed His-59 and His-251 (according to the human CD39 sequence) as equally possible targets of the inactivating DEPC modification. Potato apyrase lacks a homologue for the former residue, while the latter is preserved in the enzyme sequence. Therefore, this enzyme was exposed to DEPC, and since hydrolysis of ATP and ADP by potato apyrase was insensitive to modification with DEPC, it was concluded that His-59 is the essential residue in CD39 that is affected by DEPC modification in a way that causes inactivation of the enzyme. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Ecto-ATPDase; Ecto-ATPase; Apyrase; CD39; Diethyl pyrocarbonate; Essential histidyl residue

1. Introduction

E-type ATPases are enzymes that hydrolyse phosphoanhydride bonds in various nucleoside tri- and/or

diphosphates; they show an obligatory requirement for divalent cations without strict selectivity between Ca^{2+} and Mg^{2+} , and they are insensitive to commonly used inhibitors of F-type, P-type and V-type ATPases [1]. Structurally, some of the E-type ATPases originated from mutually unrelated protein families [2–8], but the largest, widely distributed group of these enzymes belongs to the CD39-like family of proteins. Lymphoid cell activation antigen CD39 and potato apyrase are prototypes for membrane-bound and soluble forms, respectively, of members of this family [2,9,10] which includes a broad variety

Abbreviations: ATPDase, ATP diphosphohydrolase; EBV-B cells, Epstein–Barr virus-transformed lymphoblastoid B cells; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PSS, physiological salt solution; HB, HEPES buffer supplemented with NaCl, CaCl_2 and glucose

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of enzymes expressed from protozoa and plants to mammals. Membrane-bound forms are found preferentially, but not exclusively, in the plasma membrane, where the catalytic site of the protein is located at the extracellular side. Members of the family differ in substrate specificity and in ability to hydrolyze nucleoside di- and triphosphates. Some members, like the enzymes from chicken gizzard [11] and rat brain [12], are ATPases, exhibiting a high selectivity to nucleoside triphosphates only, while e.g. CD39s and potato apyrase are ATP diphosphohydrolases (ATPDases), or apyrases, hydrolysing nucleoside di- and triphosphates almost equally well [9,13,14]. Still other members of the family, like the chicken oviduct enzyme [15] and HB6 from human brain [16], show an intermediate selectivity. It has not yet been unambiguously established how the differences in substrate specificity relate to structural differences, but it is noteworthy that cells may co-express these enzymes [12], indicating that they may differ with respect to function.

Comparison of sequences revealed that CD39-like proteins all contain five apyrase conserved regions (ACRs) [2,17,18]. Analysis of these regions disclosed certain motifs that are conserved also in functionally important regions of proteins that belong to the well-characterised actin/hsp70/sugar kinase superfamily [2]. In CD39-like proteins, site-directed mutagenesis of residues with established function in the actin/hsp70/sugar kinase superfamily is a promising approach to elucidate critical residues and structural elements involved in the enzymatic function [17,19]. An alternative approach in the search for functionally important regions and residues may be provided by a selective chemical modification.

Diethyl pyrocarbonate (DEPC) is a widely used tool in chemical modification of proteins because of the high selectivity of the reagent to histidyl residues [20]. Even though 'silent' modifications not affecting the enzymatic function may take place, modification with DEPC was successfully used in studies of various groups of enzymes, e.g. ribonucleases, proteases and P-type ATPases [20–22]. In studies on various E-type ATPases, inactivation of the enzyme by DEPC was first demonstrated for rabbit and chicken transverse tubule ATPases, and histidyl residues were suggested as the target of the inactivating modification [23,24]. Recently, inactivation of E-type ATPases in

cardiac, renal and intestinal rat membranes, as well as in chicken gizzard and liver membranes, has been demonstrated [25,26], but sequence information [11] is available for only one of these DEPC-sensitive enzymes [26].

This paper is focused on selecting for DEPC modification, by search among cell types and species, some CD39-like proteins that contain sequences that may provide information enabling distinction between different possible targets of the enzyme-inactivating modification. In this way, human CD39, rat CD39 and potato apyrase were selected. By comparing the inactivation results with the corresponding enzymes' sequences, evidence was obtained indicating that His-59, according to the sequence of human CD39, is the most plausible target of modification by DEPC. This residue seems to have an important functional significance in the enzymatic catalysis.

2. Materials and methods

2.1. Materials

RPMI 1640 medium was obtained from Gibco Laboratories, USA, foetal calf serum was from Biological Industries, Israel, [γ - 32 P]ATP was from Amersham, isopropanol and extra-pure ethanol were from Merck. All other reagents were supplied by Sigma. Potato apyrase was grade V from Sigma that exhibits hydrolysis of both ATP and ADP.

2.2. Enzyme sources

In studies on membrane-bound enzymes, three different well-established experimental systems were used that had in common CD39 expression: (1) Epstein–Barr virus-transformed lymphoblastoid B cells (EBV-B cells) where human CD39 was first discovered and best characterised [2,9,10], (2) rat lung membranes, and (3) mesenteric small arteries, both enriched in CD39, and the latter allowing repeated treatment and assay of the same material. EBV-transformed lymphoblastoid cells JY were grown in a RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum and antibiotics, and used in the logarithmic phase of growth. Before ex-

periments, the cells were sedimented at $500\times g$ for 7 min and washed twice in a 5-fold volume of a medium, HB, consisting of (mM): *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES) 20, glucose 10, NaCl 150, CaCl_2 2, pH 7.5.

Rat mesenterium was isolated from male Wistar rats and placed in ice-cold physiological salt solution, PSS, pH 7.5, containing, mM: HEPES 5, NaCl 120, KCl 4.6, KH_2PO_4 1.2, MgSO_4 1.2, EDTA 0.026, NaHCO_3 25, glucose 5.5 and CaCl_2 2.5. Segments of vessels were dissected free of fat and connective tissue. Before incubation and assay of ecto-ATPase activity in PSS, the segments were weighted [27,28].

The post-mitochondrial membrane fraction from rat lungs was isolated from tissue, homogenised in 30 mM histidine, pH 7.4, containing 7.7% sucrose and 0.1 mM EDTA, by differential centrifugation (at $10000\times g$ for 15 min and centrifugation of the supernatant at $100000\times g$ for 1 h). The resulting pellet was resuspended in the same solution, but histidine was replaced with HEPES, 30 mM, and the specific activity determined to be 4.6 U per mg of protein. Protein was measured according to the Coomassie brilliant blue technique [29] using BSA as a standard.

2.3. Modification by DEPC

Serial dilutions of DEPC were prepared in dry ethanol and the actual DEPC concentration was determined by reaction with 10 mM imidazole [20]. EBV-B cell samples, suspended in HB, were placed on ice for 5 min, and DEPC in ethanol or ethanol alone (5% final concentration) was added to the samples. After 20–30 min at 4°C , the modification was terminated by adding a 5-fold molar excess over DEPC of histidine buffer, pH 7.5. The concentration of cells during DEPC treatment was $2.5\text{--}5\times 10^5$ cells/ml. When DEPC modification in the presence or absence of 2.5 mM UTP was compared, more dilute samples (2×10^5 cells in 2 ml of HB) were used, so that the decrease in substrate concentration due to enzymatic hydrolysis did not exceed 5%. After modification, samples were washed twice with HB. When studying the pH-dependence of DEPC inactivation, cells were washed in HB buffer adjusted to pH 6.0, 6.5, 7.0 or 7.5, suspended in HB of the corresponding

pH and DEPC modification was performed as above. Reversal of DEPC inactivation was studied using EBV-B cells modified at pH 7.5. After washing and resuspending in HB, the cells were treated with 200 mM hydroxylamine, added from a 1 M stock solution of hydroxylamine in HB, adjusted to pH 7.5. The mixture was incubated at 4°C for 10–30 min, diluted 5-fold by HB, and cells were sedimented and washed, as above. Rat artery segments were treated with DEPC in PSS at 4°C for 30 min, and the rat lung membrane preparation was modified under the same conditions, but in HEPES buffer (30 mM, pH 7.4), and the protein concentration during modification was 43 $\mu\text{g}/\text{ml}$. The control and the modified membrane preparations were diluted 1000 times in the assay. In the experiment (Fig. 6) showing protection by UTP from DEPC inactivation, 2.5 nmol of UTP was added to each assay with the enzyme source. This could be taken into account calculating the concentration and the specific radioactivity of the combined substrate, ATP and UTP, since it was shown [30] that both nucleotides serve equally well as substrates for the rat lung enzyme. Potato apyrase was dialysed against HB prior to DEPC modification.

2.4. Determination of nucleotide hydrolytic activity

Ecto-ATPase activity of EBV-B cells and rat mesenteric arteries, as well as E-type ATPase activity of the rat lung membrane fraction were determined as the rate of enzymatic P_i release from nucleoside tri- or diphosphate. Control and DEPC-treated EBV-B cells were suspended in HB. Aliquots were drawn for cell counting in a Coulter Multisizer II (Counter Electronics, Northwell Drive, UK). Routinely, the standard assay was performed in 96-well plates for tissue cell culture (Greiner). Duplicate samples of 0.05–0.1 ml of cell suspension (10^4 cells) were added to preheated HB containing sodium orthovanadate, oligomycin and ATP, so that the final concentrations in the assay were 0.1 mM, 10 μM and 1 mM, respectively, in 0.3 ml total volume. Plates were covered by a plate sealer and incubated in a water bath at 37°C during 15–30 min. The reaction was terminated by placing the plate on ice and adding 30 μl 6 N H_2SO_4 . Samples were centrifuged for 3 min at $400\times g$, and the amount of P_i released from ATP was determined

in the supernatants using two colourimetric procedures of different sensitivity [31,32], modified as described previously [4]. Two values, non-enzymatic P_i release from ATP into the assay medium during incubation without cells and the P_i content in samples incubated without ATP, were subtracted from the total P_i release giving values for the enzymatic ATP hydrolysis. KH_2PO_4 was used as a standard. Ecto-ATPase activity of the EBV-B cells (in a logarithmic phase of growth) was 67 ± 14 mU/ 10^6 cells. The assay for ADP hydrolysis contained additionally 0.2 mM diadenosine pentaphosphate. The actual concentrations of all nucleoside di- and triphosphates were determined spectrophotometrically, and the enzymatic hydrolysis of ADP, GTP, CTP and UTP was measured according to the procedure described for ATP.

For measuring the ecto-ATPase activity of rat small mesenteric artery, each segment of rat mesenteric artery (average weight 2 mg per segment) was preincubated at 37°C for 2 min in 450 μ l of PSS. Fifty μ l of 10 mM [32 P]ATP (specific activity 600 cpm per nmol) was added, and hydrolysis allowed to proceed at 37°C for 10 min. At the end of the assay, the segment was removed/transferred and the incubation medium placed on ice. Upon the addition of 50 μ l of ice-cold 50% TCA, $^{32}P_i$ was extracted and determined according to [33]. Corresponding to each set of conditions, [32 P]ATP hydrolysis was measured in incubations without vessel and the value subtracted as blank [27,28]. Average ecto-ATPase activity was 3.9 U/g of tissue.

The E-type ATPase activity of rat lung membrane preparation was measured in an assay volume of 0.5 ml, and the assay medium consisted of, μ M: Mg^{2+} 2, sodium orthovanadate 50, oligomycin 6, NEM 500 and levamisole 1000, in 30 mM HEPES buffer (pH 7.4 at 37°C) with 20 mM of NaCl. After 10 min at 37°C, the reaction was stopped adding 50 μ l of ice-cold 50% TCA on ice, and released P_i was determined according to [33]. In the standard assay (Fig. 2), the concentration of Ca^{2+} and [32 P]ATP was 50 μ M, whereas Ca^{2+} and [32 P]ATP varied in parallel in the kinetic experiment shown in Fig. 6. In both cases, the specific activity of [32 P]ATP was $2\text{--}4 \times 10^4$ cpm/nmol and the quantity of enzyme added between 0.01 and 0.2 mU [27,28].

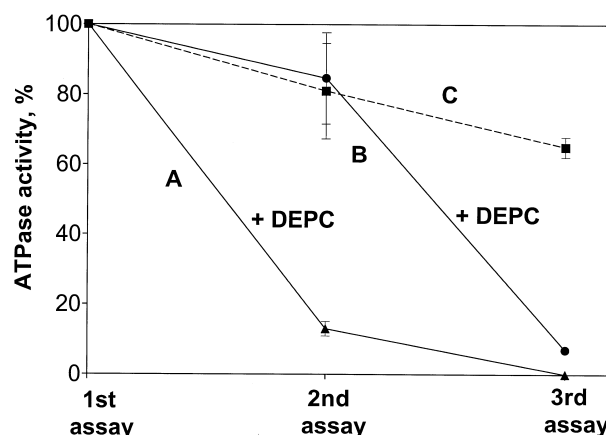


Fig. 1. Inactivation of ecto-ATPase activity of mesenteric artery segments by DEPC modification. The ecto-ATPase activity of nine segments was determined in PSS with 1 mM [γ - 32 P]ATP (1st assay), and in the following 30 min, three segments (group A, ▲) were incubated with DEPC (0.5 mM) in PSS, while six segments (group B, ●, and C, ■) were incubated as control, at 4°C. At the end of the incubation, each of the nine segments was assayed again (2nd assay). During the next 30 min, another three segments (group B) were incubated with DEPC (0.5 mM), while the six segments in group A and C were incubated as control, at 4°C. Finally, all nine segments were submitted to the 3rd assay of ecto-ATPase activity. Corresponding to each transfer, from assay to incubation and back, the segments were thoroughly rinsed in PSS. Remaining ecto-ATPase activity in the 2nd and 3rd assay is expressed as percent of the activity measured in the 1st assay of the individual segment. (The mean of the latter value was 3.9 U/g of tissue).

3. Results

The ecto-ATPase activity of rat small mesenteric arteries was previously characterised in details [27,28]. The enzyme belongs to the CD39 family [34].

In Fig. 1 are shown the results obtained when the ecto-ATPDase of rat small arteries was modified by DEPC. This enzyme source enables a quantitative transfer, repeated treatment and repeated assay of the same material: the ecto-ATPase activity of nine segments was determined (first assay), and in the following 30 min, three segments (group A) were incubated with DEPC (0.5 mM), while six segments (group B and C) were incubated as control, at 4°C. At the end of the incubation, each of the nine segments was assayed again (second assay). During the next 30 min, another three segments (group B) were incubated with DEPC (0.5 mM), while the six segments in group A and C were incubated as control,

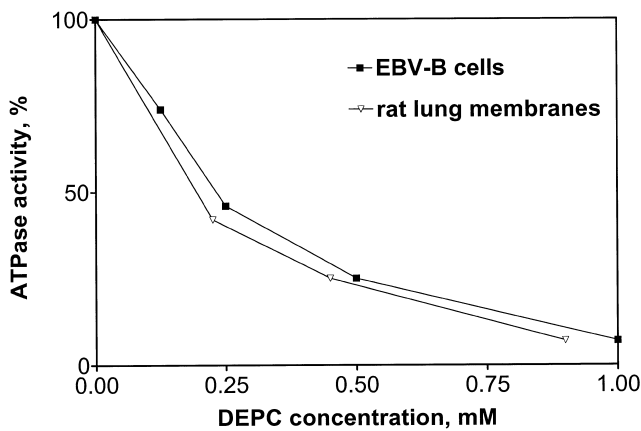


Fig. 2. Inactivation by modification with DEPC of the ecto-ATPase activity in human EBV-B cells and of E-type ATPase activity in rat lung membranes. EBV-B cells (■) and rat lung membranes (△) were incubated with the indicated DEPC concentrations at 4°C at pH 7.5 in HB or HEPES (30 mM, pH 7.4), respectively. After 30 min, the reaction was terminated by adding a 5-fold molar excess of histidine, pH 7.5, over DEPC. The remaining activity was measured in the standard assay of each enzyme source and expressed in percent of the initial activity.

at 4°C. Finally, all nine segments were submitted to the third assay of ecto-ATPase activity. Corresponding to each transfer, from assay to incubation and back, the segments were thoroughly rinsed in PSS. Remaining ecto-ATPase activity in the second and third assay is expressed as percent of the activity measured in the first assay of the individual segment. (The mean of the latter value was 3.9 U/g of tissue). The decline in ecto-ATPase activity in the controls (group B: between first and second assay, and group C: between first and second and between second and third assay) is caused by substrate inactivation during the assay period(s) [1,27]. DEPC treatment of group A resulted in a strong decrease in the enzymatic activity to 13% of the initial level, while the control vessels (groups B and C) retained 83% of the initial ecto-ATPase activity. Thus, in comparison to the control, activity dropped to 15.7%. After DEPC treatment of group B, the ecto-ATPase activity was reduced to 7% of the initial level, or to 11% if compared to the control (group C) that fell to 65% of the initial level of activity. No recovery of the enzymatic activity (between second and third assay) could be observed in the vessels of group A, indicating that the modification was stable and recycling of the en-

zyme was not observed. Thus, DEPC modification strongly inactivates ecto-ATPase activity of rat mesenteric small arteries, and the degree of substrate-dependent inactivation, that precedes DEPC-treatment, does not seem to influence the sensitivity of the enzyme to DEPC inactivation.

CD39/ecto-ATPDase was first discovered in EBV-B cells, where it was characterised as the only enzyme hydrolysing extracellular ATP [9]. In Fig. 2 are shown the results of DEPC modification of these cells, as well as of rat lung membranes, known to highly express CD39 [12,34]. The ecto-ATPase activity of EBV-B cells and the E-type ATPase activity of rat lung membranes are inactivated to the same extent by DEPC (Fig. 2), and the sensitivity of these enzymes to DEPC is comparable with that of the enzyme in rat mesenteric small arteries (Fig. 1). Since CD39 is capable of hydrolysing various nucleoside di- and triphosphates, we next tested the substrate specificity of DEPC-inactivated CD39 (Fig. 3). Modification by DEPC, terminated by excess of histidine, led to inactivation of all the enzymatic activities of CD39 in EBV-B cells: ADP hydrolysis was reduced

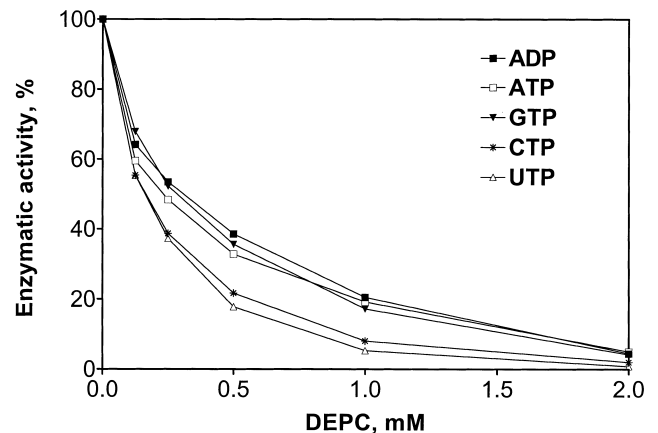


Fig. 3. Inactivation by DEPC treatment of nucleotide hydrolysis catalysed by ecto-ATPDase of EBV-B cells. For 20 min, EBV-B cells were incubated (see legend to Fig. 2) with the indicated DEPC concentrations, then the hydrolytic activity was measured in aliquots of the cell suspension and expressed in percent of the initial activity in each experiment. The hydrolytic activity was measured in HB supplemented with 0.1 mM orthovanadate and 10 μ M oligomycin using the indicated nucleotides at 1 mM as substrates. The control activity in hydrolysis of ATP, ADP, GTP, CTP and UTP was 67.8 ± 2.0 , 48.9 ± 1.6 , 62.7 ± 2.2 , 66.5 ± 1.2 , 73.7 ± 1.9 mU/ 10^6 cells, respectively (mean \pm S.E.M. of three independently grown cell cultures, duplicate samples).

to the same extent as ATP hydrolysis, but the hydrolysis of pyrimidine nucleoside triphosphates was suppressed by DEPC modification slightly more than hydrolysis of purine nucleoside triphosphates (Fig. 3).

DEPC has a high selectivity to histidyl residues, but it is capable of modifying also tyrosyl, lysyl, arginyl and cysteinyl residues [20]. Two criteria are commonly used to distinguish between the possible target residues [20]: (1) the pH-dependence of the modification: this is determined by pK of the side chain group of the residue, and (2) reversibility of the modification by treatment with hydroxylamine, which is rapid in the case of carbethoxylated histidyl, but is also possible if a tyrosyl residue is modified. Fig. 4a shows the relationship between DEPC inactivation of ecto-ATPase in EBV-B cells and pH during DEPC modification. At all tested pH values, the inactivation follows a single exponential dependence on the concentration of the modifying reagent, and the obtained pH profile is most consistent with carbethoxylation of the imidazole group in histidyl residue [20] as has been proposed formerly [23,24,26]. This conclusion is further supported by the rapid reversibility of the enzyme inactivation upon treatment with 200 mM hydroxylamine, consistent with decarbethoxylation of modified histidyl residues (Fig. 4b). At low DEPC concentrations, recovery of the enzymatic activity is almost complete in 10 min if compared to the activity of control EBV-B cells treated only with 200 mM hydroxylamine. Restoration was 82% and 76% for the preparations modified with 1 mM and 2 mM DEPC, respectively (Fig. 4b). Recovery of the enzymatic activity did not increase if the treatment with hydroxylamine was extended to 30 min (not shown). Incomplete recovery of the activity could be the result of a concomitant modification of another, not histidyl, target residue. The increase in the fraction of irreversibly inactivated ATPase with increase in DEPC concentration may, however, alternatively point to a formation of a di-substituted histidyl derivative. Such a compound is known to be insensitive to treatment with hydroxylamine [20].

Inactivation of the ecto-ATPase activity in EBV-B cells could be prevented if the substrate of the enzyme was present during DEPC treatment (Fig. 5). In the presence of 2.5 mM UTP, protection of the

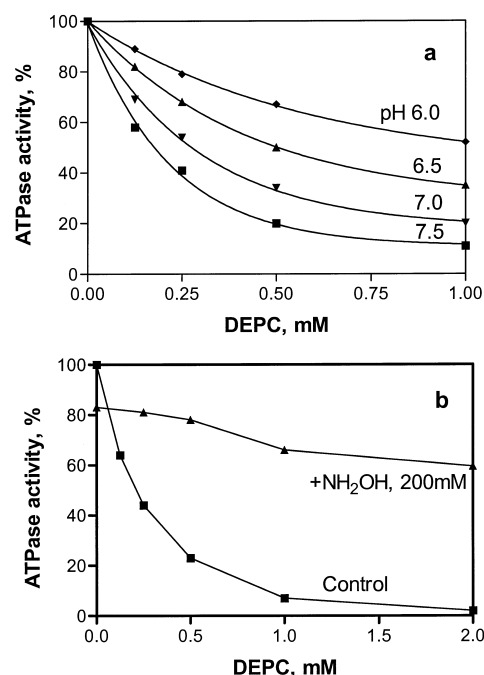


Fig. 4. pH-dependence of DEPC-induced inactivation of the ecto-ATPase activity of EBV-B cells (a) and (b) reversal of the inactivation by hydroxylamine. (a) Cells were washed with HB adjusted to pH 6.0, 6.5, 7.0 or 7.5. DEPC modification was performed at the same pH; (b) after modification at pH 7.5 with the indicated DEPC concentrations, cells were sedimented, suspended in HB and divided into two portions. One was kept without treatment, while hydroxylamine from a stock 1 M solution, pH 7.5, in HB was added to the other (final concentration 200 mM). After incubation for 10 min at 4°C, all suspensions were diluted 5-fold in HB, washed twice, sedimented, resuspended and counted. Aliquots were drawn for measuring the ecto-ATPase activity of the cells treated with hydroxylamine after DEPC modification (\blacktriangle) or left without treatment after the modification (\blacksquare).

enzyme was observed at all DEPC concentrations tested, but protection was most effective at a higher ratio of substrate over modifier concentration. Thus, while more than half of the enzymatic activity was lost during modification with 0.2 mM DEPC alone, inactivation was prevented almost completely by the presence of 2.5 mM UTP during DEPC treatment (Fig. 5). In rat mesenteric small vessels, the presence of substrate (UTP) prevented inactivation of the ecto-ATPase by DEPC (not shown), but since the presence of substrate alone causes inactivation of the enzyme (decreased substrate affinity) in intact rat vessels [27], the interpretation of these experiments is difficult. Protection from DEPC inactivation of

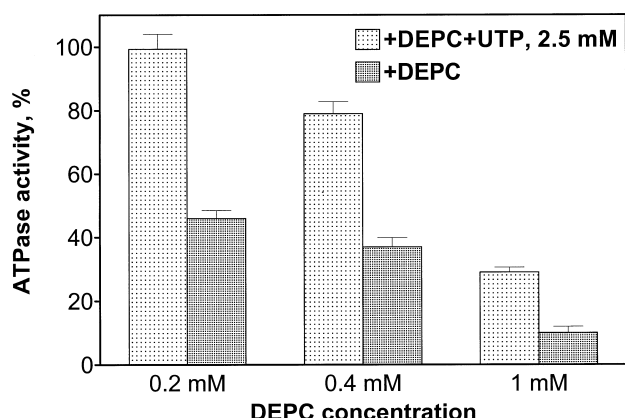


Fig. 5. Protection against DEPC inactivation by the substrate of EBV-B cell ecto-ATPase activity. Cells were incubated at 4°C in HB with the indicated DEPC concentrations, in the absence (dark columns) or the presence (light columns) of 2.5 mM UTP. After 20 min incubation, cells were washed, counted and the ecto-ATPase activity determined. The enzymatic activity remaining after modification is expressed as percent of the activity of the control sample containing all the added compounds except DEPC (mean \pm S.E.M. of five experiments).

E-type ATPase activity of rat lung membranes could be demonstrated (Fig. 6), and also in this enzyme source was the protective effect dependent on the ratio between substrate and modifier concentration (not shown). Since pyrimidine nucleotides, unlike purines [20], do not react directly with DEPC, it is binding of the substrate to the enzyme that prevents modification of a functionally essential histidyl residue due to a steric hindrance or a conformational transition in the protein, which affects either reactivity or accessibility of the target. Fig. 6 shows double reciprocal plots for E-type ATPase of rat lung membranes in control preparations and preparations modified by DEPC in the absence or presence of UTP. After modification, samples were 1000-fold diluted in the assay where ATPase activity was determined by measuring radioactive phosphate release from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, keeping an equimolar ratio of Ca and ATP over a broad range of substrate concentrations. DEPC modification decreased the maximal velocity of ATP hydrolysis, and the presence of UTP during DEPC treatment prevented this inactivation of the enzyme (Fig. 6). A decrease in the maximal velocity was obtained also when comparing hydrolysis of UTP by ecto-ATPase of DEPC-modified and control EBV-B cells (not shown). The observed kinetic patterns are compatible with an impaired cata-

lytic step or complete inactivation of the part of the molecules, while non-modified molecules bind and hydrolyse substrate as before [35].

Search for a possible target of DEPC modification should focus on histidyl residues conserved in the CD39 family of proteins, and comparison of sequences of membrane-bound and soluble members revealed only five conserved histidyl residues to be considered. The regions of the polypeptide chains, which include these conserved histidyl residues, are presented in Table 1. The conserved histidyl residue found closest to the C-terminal, His-474, is preserved only in all CD39s, but not in other enzymes belonging to the CD39 family of proteins. Since chicken gizzard ecto-ATPase lacks this residue, and since DEPC was shown to inactivate this enzyme activity [26], His-474 could not be the target of the inactivating modification. For the same reason, His-438 could be excluded, and His-121 was discarded as a possible candidate, since this residue is absent in rat CD39 and chicken oviduct ATPase, and since the former enzyme was shown to be DEPC-sensitive in the present work. The remaining two conserved histidyl residues were His-251, found in all the discussed sequences (Table 1), and His-59, that is missing in HB6 and potato apyrase.

Thus, the sensitivity of potato apyrase to DEPC

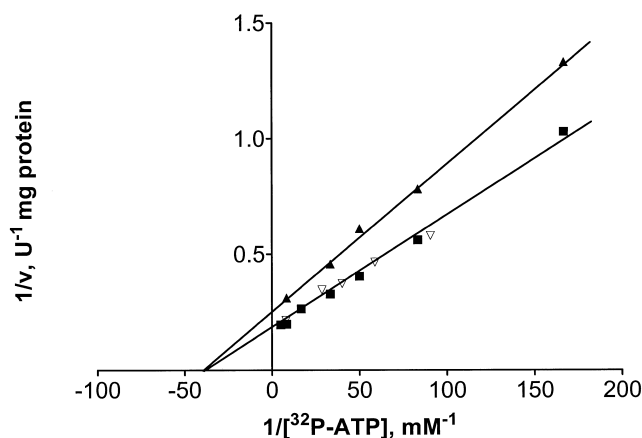


Fig. 6. Double reciprocal plots of substrate curves of the E-type ATPase activity of rat lung membranes. Control preparations (■), enzyme-treated with 0.2 mM DEPC (▲) and enzyme-treated with 0.2 mM DEPC in the presence of 5 mM UTP (▽). In the latter case, 2.5 nmol of UTP was added to each assay with the enzyme source. This was taken into account calculating the concentration and the specific radioactivity of the combined substrates ATP and UTP.

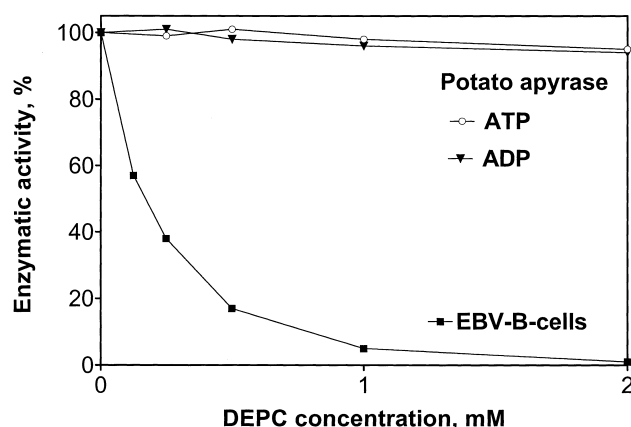


Fig. 7. Effects of DEPC modification of EBV-B cell ecto-ATPase activity and of potato apyrase E-type ATP(D)ase activity. Potato apyrase, dialysed against HB, and EBV-B cells were treated with DEPC in the indicated concentrations at 4°C during 30 min. ATP hydrolysis by EBV-B cells (■) and ATP (○) and ADP (▼) hydrolysis by potato apyrase were measured using aliquots of the samples and expressed as percent of the control activity of the individual enzyme.

inactivation was used to distinguish between His-59 and His-251 as possible targets of DEPC inactivation. As shown in Fig. 7, there is a drastic difference between the enzymatic activities of human CD39 and potato apyrase in their sensitivity to DEPC inactivation. In control experiments, stabilisation of CD39 oligomers in EBV-B cells by cross-linking at pH 8.8 with 10 mM dimethyl pimelimidate [4], either in the

absence or the presence of ATP, did not result in the occurrence of a DEPC-insensitive fraction of ecto-ATPase (not shown). Since the ATPase and the ADPase activity of potato apyrase were insensitive to DEPC modification, this points to His-59 as the most likely target of DEPC inactivation.

4. Discussion

Identification of residues that are essential for the catalytic function of CD39-like proteins contributes importantly to the elucidation of the underlying reaction mechanism. Recent cloning and sequencing of a number of these proteins has led to the discovery in their structure of certain motifs conserved in members of the actin/hsp70/sugar kinase superfamily [2,17]. Functional knowledge about residues in these motifs was used to select targets for site-directed mutagenesis [19]. In the present work, inactivation of the enzymes caused by chemical modification by DEPC was analysed in the context of known primary structure and used as an independent alternative approach to search for regions and residues that are functionally important among CD39-like proteins, while not obligatorily conserved among other members of the superfamily. DEPC modifications of human CD39, rat CD39 and potato apyrase were evaluated in the perspective of previous studies [23,24,26]

Table 1

Conserved His-containing regions selected from multiple alignment of the complete sequences of membrane-bound E-type ATPases and potato apyrase

	1	2	3	4	5
Human CD39	DAGSSHTS 61	SQHQETPVYLGAT 131	VYTHSFLC 255	HIHFIGK 442	TPLSHSTY 477
Rat CD39	DAGSSHTN 61	SKQHQTTPVYLGAT 131	VYTHSFLC 254	QIHFMGK 443	PPLPHSTY 478
Mouse CD39	DAGSSHTN 61	SKHHQTTPVYLGAT 131	VYTHSFLC 254	QIHFMGK 442	PPLPHSTY 477
Bovine CD39	DAGSSHTS 61	IQHMETPVYLGAT 131	VYTHSFLC 255	NIHFMNK 445	PPLPHSTY 480
Human CD39L1	DAGSSHTS 52	ERHAGTPLYLGAT 122	VYTHSFLC 242	GVIFQKK 405	KGTFDFSSW 441
Rat brain ecto-ATPase	DAGSSHTS 52	DRHASTPLYLGAT 122	VYTHSFLC 242	EVVFQKK 428	KGTHFSSW 464
Ch.giz. ecto-ATPase	DAGSSHTA 50	EKHADTPLYLGAT 120	VYTHSFLC 239	SIAFQKK 429	RSMLYNW 465
Ch.ov. ecto-ATPDase	DAGSTHTA 53	EQQWQTPTYLAT 123	LYTHSYLC 244	NIHFSQK 430	KGHEPSLW 468
Human HB6	DAGSSRTT 69	HLHGSTPIHLGAT 139	LYTHSFQC 261	QIHFEKE 451	LPIEPPVF 487
Potato apyrase	DAGSTGSR 48	ELQSETPLELGAT 127	LYVHSYLN 246	TVIHVDQ 423	S— — — 454

Alignment was performed using the ClustalW program. The sequences and GenBank accession numbers are, respectively, human CD39, S73813; rat CD39, U81295; murine CD39, AF037366; bovine CD39, AF005940; human CD39L1, U91510; rat brain ecto-ATPase, Y11835; chicken gizzard ecto-ATPase; U74467, chicken oviduct ecto-ATPDase, AF041355; human HB6, AF034840; and potato apyrase, U58597. The numbers on the right side of each of five columns indicate numbers of the C-terminal residue of the fragment in each of the individual sequences.

leading to the conclusion that the target of inactivating DEPC modification is His-59 in human CD39 and homologous residues in other CD39-like E-type ATPases.

First, inactivation by DEPC of the enzyme in rat mesenteric small arteries, rat lung membranes and in human EBV-B cells was demonstrated (Figs. 1 and 2). It was further shown that all the enzymatic activities expressed by CD39 were inactivated by DEPC modification (Fig. 3). Characteristics of DEPC inactivation, pH profile and reversibility by hydroxylamine treatment (Fig. 4a,b), indicated that the inactivating DEPC modification resulted in carbethoxylation of histidyl residue(s), as has been proposed previously for some other E-type ATPases [23,24,26]. Next, it was shown that the presence of the substrate, UTP, protected the enzyme from DEPC inactivation (Figs. 5 and 6). ATP may react directly with DEPC [20], and the protective effect of this substrate [23,26] could be caused by a decrease in the actual DEPC concentration in the presence of ATP. But the choice of a pyrimidine substrate eliminated this possibility, and the protective effect of UTP against DEPC inactivation (Figs. 5 and 6) indicated that a region of the enzyme containing a critical histidyl residue was protected from DEPC by substrate binding, possibly because the binding of substrate was followed by a local function-associated conformational transition that prevented DEPC modification of the target residue. On the other hand, when the target histidyl residue was modified by DEPC, the enzyme molecule became inactive. The introduction of an additional bulky side group probably represented a steric disadvantage that prevented either substrate binding or a conformational transition involved in catalysis, or both. It is consistent with the decrease in maximal velocity seen in Fig. 6 that a fraction of the enzyme molecules had become inactive, the rest catalysing the hydrolysis of the substrate as usual. Increasing the substrate concentration did not change the proportion between the two fractions (Fig. 6).

Search for the residue critical for DEPC inactivation (by comparing sequences of CD39-like proteins and the corresponding DEPC sensitivity) led us to conclude that the most probable target of DEPC inactivation is the conserved His-59, according to the sequence of human CD39. It is noteworthy that

this essential target residue is localised within ACR1, i.e. one of the two ACR sequences that are conserved in the actin/hsp70/sugar kinase superfamily [2]. The functional significance of ACR1 and two residues within this region, homologous to Asp-54 and Gly-56 in CD39, has been demonstrated by deletions and site-directed mutagenesis in human CD39 and in HB6, respectively [17,19]. Furthermore, these two amino acid residues that are both present in the β -phosphate binding motif of all members of the actin/hsp70/sugar kinase superfamily were shown to participate in the enzymatic catalysis in various members of the superfamily [19,36]. The inactivation of CD39 by modification of His-59, localised in close proximity to these two critical residues, demonstrates further the functional significance of ACR1.

In one of the membrane-bound CD39-like ecto-ATPases, HB6, His at this position is substituted by Arg [16]. This may indicate that either the residue, even though being part of the sequence in the catalytic region, is not functionally significant, and DEPC modification inactivates the enzyme solely because of steric factors, or that the function carried by the residue at this position tolerates some substitutions. Noteworthy, only HB6 sequence contains a histidyl residue (marked in bold in Table 1) in a very conserved part of ACR2. Since the ACRs are assumed to be in a close proximity in the tertiary structure [2], this histidyl could be a structural-functional substitution for histidyl residue lacking in ACR1 of HB6. Chemical modification data do not prove directly functional significance and the role of the particular target of the modification, but a possible function can be suggested based on conservation of the residue in a number of related sequences, as well as by analogy with similar proteins. There are two functionally important histidyl residues in the structure of actin [36]. One of them, His-73, is located close to ACR1 and, according to atomic structure data, it occupies a position where it participates in the enzymatic catalysis by coordinating the water molecule that attacks the γ -phosphate of ATP during the enzymatic catalysis [36]. In hsp70, the same position is occupied and the same function undertaken by Lys-71 [36], indicating no strict requirement for His in the specific function. Potato apyrase is DEPC-insensitive (Fig. 7). This enzyme contains Gly (Table 1) at the position homologous to His-59 in the CD39

sequence. If by analogy with actin [36], His-59 has a functional significance in E-type ATPases, (1) this function can tolerate some amino acid substitutions, less dramatic than introducing a hydrophobic carboxy group into His; (2) structural limitations or requirements put on the enzyme by this function (which could be, for example, coordination of the water molecule attacking γ -phosphate) may differ for highly organised membrane-bound oligomer and soluble monomeric enzyme.

In conclusion, His-59 in the sequence of CD39 and homologous sequences in CD39-like proteins is the most probable target for inactivation by DEPC. Further studies are required to elucidate the molecular mechanism behind the inactivation and roles for this histidyl residue in the enzymatic function of E-type ATPases.

5. Note added in proof

While this paper was under review, A. Grinthal and G. Guidotti published results obtained by site directed mutagenesis [Biochemistry 39 (2000) 9–16]. The results indicate that His-59 has a functional role in ATP hydrolysis by CD39.

Acknowledgements

This work was supported by 'Fonden til Lægevidenskabens Fremme'. We thank Drs Alexei Kirkin, Igor Plesner and Prof. Jesper Zeuthen for support and expertise in cell culture and for helpful discussions. Birgit Sørensen is gratefully acknowledged for her excellent technical assistance. A preliminary version of this work has been presented at the Second International Workshop on ecto-ATPases, June 14–18, 1999, Belgium.

References

- [1] L. Plesner, Ecto-ATPases: identities and functions, *Int. Rev. Cytol.* 158 (1995) 141–214.
- [2] M. Handa, G. Guidotti, Purification and cloning of a soluble ATP-diphosphohydrolase (apyrase) from potato tubers (*Solanum tuberosum*), *Biochem. Biophys. Res. Commun.* 218 (1996) 916–923.
- [3] K. Dzhandzhugazyan, E. Bock, Demonstration of $(\text{Ca}^{2+}$ - Mg^{2+})-ATPase activity of the neural cell adhesion molecule, *FEBS Lett.* 336 (1993) 279–283.
- [4] K. Dzhandzhugazyan, E. Bock, Demonstration of an extracellular ATP-binding site in NCAM functional implications of nucleotide binding, *Biochemistry* 36 (1997) 15381–15395.
- [5] D.E. Champagne, C.T. Smartt, J.M. Ribeiro, A.A. James, The salivary gland-specific apyrase of the mosquito *Aedes aegypti* is a member of the 5'-nucleotidase family, *Proc. Natl. Acad. Sci. USA* 92 (1995) 694–698.
- [6] J.G. Valenzuela, R. Charlab, M.Y. Galperin, J.M. Ribeiro, Purification, cloning, and expression of an apyrase from the bed bug *Cimex lectularius*. A new type of nucleotide-binding enzyme, *J. Biol. Chem.* 273 (1998) 30583–30590.
- [7] R. Betto, L. Senter, S. Ceoldo, E. Tarricone, D. Biral, G. Salvati, Ecto-ATPase activity of α -sarcoglycan (adhalin), *J. Biol. Chem.* 274 (1999) 7907–7912.
- [8] M.Y. Galperin, D.R. Walker, E.V. Koonin, Analogous enzymes: independent inventions in enzyme evolution, *Genome Res.* 8 (1998) 779–790.
- [9] T.F. Wang, G. Guidotti, CD39 is an ecto- $(\text{Ca}^{2+}$, Mg^{2+})-apyrase, *J. Biol. Chem.* 271 (1996) 9898–9901.
- [10] C.R. Maliszewski, G.J. Delespesse, M.A. Schoenborn, R.J. Armitage, W.C. Fanslow, T. Nakajima, E. Baker, G.R. Sutherland, K. Poindexter, C. Birks, The CD39 lymphoid cell activation antigen. Molecular cloning and structural characterization, *J. Immunol.* 153 (1994) 3574–3583.
- [11] T.L. Kirley, Complementary DNA cloning and sequencing of the chicken muscle ecto-ATPase. Homology with the lymphoid cell activation antigen CD39, *J. Biol. Chem.* 272 (1997) 1076–1081.
- [12] B. Kegel, N. Braun, P. Heine, C.R. Maliszewski, H. Zimmermann, An ecto-ATPase and an ecto-ATP diphosphohydrolase are expressed in rat brain, *Neuropharmacology* 36 (1997) 1189–1200.
- [13] A.J. Marcus, M.J. Broekman, J.H. Drosopoulos, N. Islam, T.N. Alyonycheva, L.B. Safier, K.A. Hajjar, D.N. Posnett, M.A. Schoenborn, K.A. Schooley, R.B. Gayle, C.R. Maliszewski, The endothelial cell ecto-ADPase responsible for inhibition of platelet function is CD39, *J. Clin. Invest.* 99 (1997) 1351–1360.
- [14] E. Kaczmarek, K. Koziak, J. Seigny, J.B. Siegel, J. Anrather, A.R. Beaudoin, F.H. Bach, S.C. Robson, Identification and characterization of CD39/vascular ATP diphosphohydrolase, *J. Biol. Chem.* 271 (1996) 33116–33122.
- [15] R.S. Strobel, A.K. Nagy, A.F. Knowles, J. Buegel, M.D. Rosenberg, Chicken oviductal ecto-ATP-diphosphohydrolase. Purification and characterization, *J. Biol. Chem.* 271 (1996) 16323–16331.
- [16] T.M. Smith, T.L. Kirley, Cloning, sequencing, and expression of a human brain ecto-apyrase related to both the ecto-ATPases and CD39 ecto-apyrases, *Biochim. Biophys. Acta* 1386 (1998) 65–78.
- [17] J. Schulte am Esch II, J. Seigny, E. Kaczmarek, J.B. Siegel,

- M. Imai, K. Koziak, A.R. Beaudoin, S.C. Robson, Structural elements and limited proteolysis of CD39 influence ATP diphosphohydrolase activity, *Biochemistry* 38 (1999) 2248–2258.
- [18] E.G. Vasconcelos, S.T. Ferreira, T.U. Carvalho, W. Souza, A.M. Kettlun, M. Mancilla, M.A. Valenzuela, S. Verjovski-Almeida, Partial purification and immunohistochemical localization of ATP diphosphohydrolase from *Schistosoma mansoni*. Immunological cross-reactivities with potato apyrase and *Toxoplasma gondii* nucleoside triphosphate hydrolase, *J. Biol. Chem.* 271 (1996) 22139–22145.
- [19] T.M. Smith, T.L. Kirley, Site-directed mutagenesis of a human brain ecto-apyrase evidence that the E-type ATPases are related to the Actin/Heat shock 70/Sugar kinase superfamily, *Biochemistry* 38 (1999) 321–328.
- [20] E.W. Miles, Modification of histidyl residues in proteins by diethyl pyrocarbonate, *Methods Enzymol.* 47 (1977) 431–442.
- [21] J.P. Tenu, C. Chelis, D.S. Leger, J. Carrette, Mechanism of an active transport of calcium. Ethoxyformylation of sarcoplasmic reticulum vesicles, *J. Biol. Chem.* 251 (1976) 4322–4329.
- [22] K. Yamasaki, T. Daiho, T. Saino, T. Kanazawa, Modification of histidine 5 in sarcoplasmic reticulum Ca^{2+} -ATPase by diethyl pyrocarbonate causes strong inhibition of formation of the phosphoenzyme intermediate from inorganic phosphate, *J. Biol. Chem.* 272 (1997) 30627–30636.
- [23] T.L. Kirley, Purification and characterization of the Mg^{2+} -ATPase from rabbit skeletal muscle transverse tubule, *J. Biol. Chem.* 263 (1988) 12682–12689.
- [24] A. Saborido, G. Moro, A. Megias, Transverse tubule Mg^{2+} -ATPase of skeletal muscle. Evidence for extracellular orientation of the chicken and rabbit enzymes, *J. Biol. Chem.* 266 (1991) 23490–23498.
- [25] T. Okada, V.S. Zinchuk, T. Kobayashi, E. GarciadelSaz, H. Seguchi, Diethyl pyrocarbonate is an inhibitor of cardiac, intestinal and renal ecto-ATPase, *Acta Histochem. Cytochem.* 30 (1997) 637–642.
- [26] C.C. Caldwell, M.D. Davis, A.F. Knowles, Ectonucleotidases of avian gizzard smooth muscle and liver plasma membranes: a comparative study, *Arch. Biochem. Biophys.* 362 (1999) 46–58.
- [27] B. Juul, M.E. Lüscher, C. Aalkjær, L. Plesner, Nucleotide hydrolytic activity of isolated intact rat mesenteric small arteries, *Biochim. Biophys. Acta* 1067 (1991) 201–207.
- [28] L. Plesner, B. Juul, E. Skriver, C. Aalkjær, Characterisation of Ca^{2+} or Mg^{2+} -dependent nucleoside triphosphatase from rat mesenteric small arteries, *Biochim. Biophys. Acta* 1067 (1991) 191–200.
- [29] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [30] L. Plesner, Solubilized E-type ATPase is released from intact rat tissues in the simultaneous presence of nucleotides and detergents, in: L. Plesner, T.L. Kirley and A.F. Knowles (Eds.), *Ecto-ATPases. Recent Progress on Structure and Function*, Plenum Press, New York, 1997, pp. 41–47.
- [31] J.M. Villalba, M.G. Palmgren, G.E. Berberian, C. Ferguson, R. Serrano, Functional expression of plant plasma membrane H^{+} -ATPase in yeast endoplasmic reticulum, *J. Biol. Chem.* 267 (1992) 12341–12349.
- [32] A.A. Baykov, O.A. Evtushenko, S.M. Avaeva, A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay, *Anal. Biochem.* 171 (1988) 266–270.
- [33] O. Lindberg and L. Ernster, in: G. Gliek (Ed.), *Methods of Biochemical Analysis*, Interscience, New York, 1956, pp. 1–22.
- [34] K. Enjyoji, J. Seigny, Y. Lin, P.S. Frenette, P.D. Christie, J.S. Esch, M. Imai, J.M. Edelberg, H. Rayburn, M. Lech, D.L. Beeler, E. Csizmadia, D.D. Wagner, S.C. Robson, R.D. Rosenberg, Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation, *Nat. Med.* 5 (1999) 1010–1017.
- [35] I.W. Plesner, The apparent K_m is a misleading kinetic indicator, *Biochem. J.* 239 (1986) 175–178.
- [36] K.M. Flaherty, S.M. Wilbanks, C. DeLuca-Flaherty, D.B. McKay, Structural basis of the 70-kilodalton heat shock cognate protein ATP hydrolytic activity. II. Structure of the active site with ADP or ATP bound to wild type and mutant ATPase fragment, *J. Biol. Chem.* 269 (1994) 12899–12907.